EVIDENCE FOR PENETRANT AND NON-PENETRANT THIOL REAGENTS AND THEIR USE IN THE LOCATION OF RAT LIVER MITOCHONDRIAL D(-)-β-HYDROXYBUTYRATE DEHYDROGENASE*

Y. GAUDEMER and N. LATRUFFE

Université de Besancon, Laboratoire de Biochimie, Faculté des Sciences et des Techniques, 25030 Besancon Cédex, France

Received 16 March 1975

1. Introduction

The implication of mitochondrial protein thiols has been postulated in several functions: coupling mechanism, transport, conformations changes, dehydrogenases, etc.. A general review concerning these topics has been published recently [1]. In order to localize these SH-groups it is necessary to evaluate the penetrability of SH reagents through the inner membrane; this was done by measuring, in the presence of different thiol reagents in isotonic conditions and varying the experimental conditions, the amount of free reduced (GSH) and oxidized (GSSG) glutathione in the matrix compartment. These results put forward two types of reagents: penetrant and non-penetrant thiol reagents. The use of these two types permits to localize the rat liver mitochondrial D(-)-β-hydroxybutyrate dehydrogenase on the inside part of the inner membrane. Non penetrant thiol reagents do not inhibit the D(-)-β-hydroxybutyrate oxidation in intact mitochondria but only in sonicated mitochondria whereas penetrant thiol reagents inhibit immediately with intact mitochondria. This location is confirmed by measuring NADH oxidation in the presence of acetoacetate with intact and sonicated mitochondria.

*These results have been presented at the meeting organized by the Société de Chimie Biologique, 'Form des Jeunes', Montpellier, 5-7 September 1974.

Abbreviations: GSH and GSSG, reduced and oxidized glutathione respectively; DSSD and DSH represent the oxidized and reduced form, respectively of Ellman reagent; G.R., glutathione reductase (EC 1.6.4.2.); TCA, trichloracetic acid; BOH, D(-)-β-hydroxybutyrate; AcAc, acetoacetate; Rot, rotenone; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; P_i, inorganic phosphate.

2. Materials and methods

Rat liver mitochondria (washed three times) were isolated in 0.25 M sucrose buffered with 1 mM Tris–HCl at pH 7.4 according to Weinbach [2]. Sonicated mitochondria were obtained by sonication (Braunsonic 300 Homogenisator) of intact mitochondria 30 sec at maximum input, temperature 4°C. The protein content is estimated using the 'quick biuret' method [3] with bovine serum albumin as reference. Reduced and oxidized glutathione were estimated by the method of Tietze [4] based on the catalytic action of GSH or GSSG in the reduction of Ellman reagent (DTNB) [5] by a mixture of NADPH and yeast glutathione reductase. The different experimental steps of the method used are shown in fig.1. Oxygen consumption was estimated polarographically using a Clark oxygen electrode (Gilson medical electronics). NADH oxidation is measured at 366 nm using an Eppendorf photometer coupled to an Eppendorf Recorder 6511. The respiratory medium is described by Ernster [6]. All chemicals used were products of the highest available grade.

3. Results and discussion

High reactive glutathione [7,8] represents main non protein-thiol in mitochondria [9,10] and may

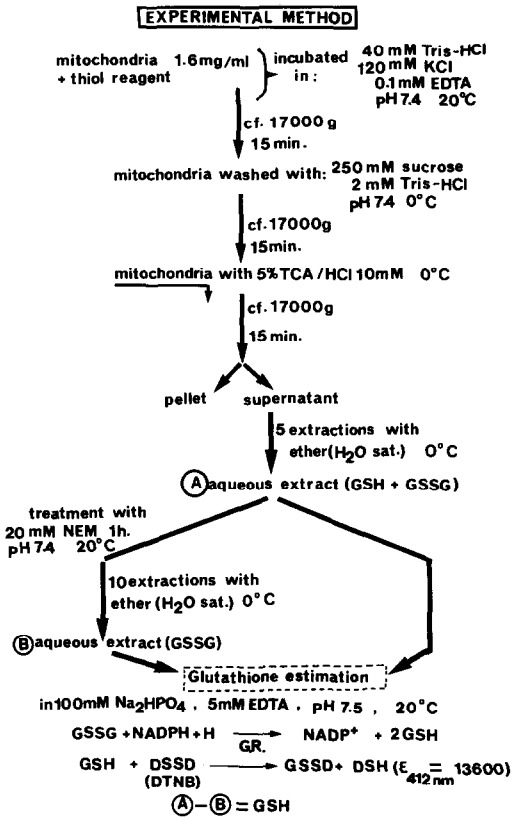

Fig.1. Experimental method for the estimation of the mitochondrial glutathione content.

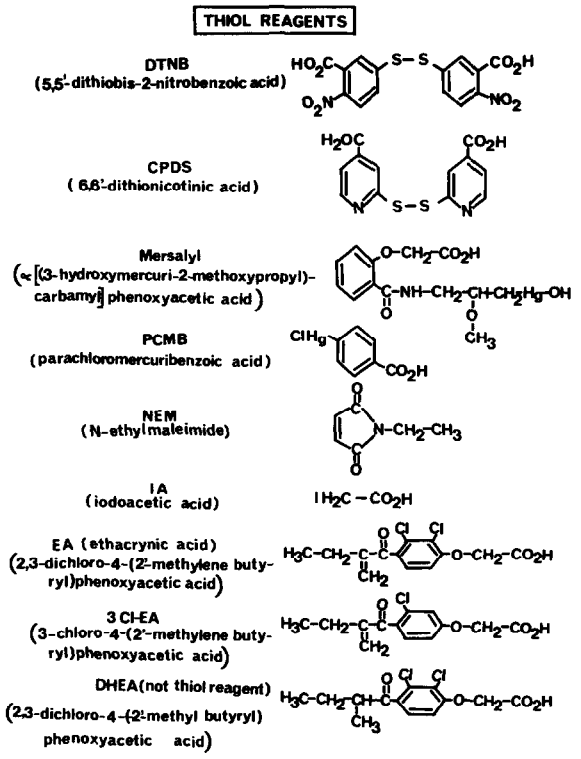

Fig.2. Different thiol reagents tested.

be a reservoir of reducing equivalents. We found that rat liver mitochondria contain GSH and GSSG (5–6 nmol and 0.1–0.4 nmol respectively per mg protein) in agreement with other results [7,8]. Using fluorometric method (not entirely specific) Jocelyn and Kamminga [11] found about twice as much. Figure 2 shows the different thiol reagents tested; disulfides: DTNB, CPDS; mercurials: PCMB, mersalyl; alkylating reagents: NEM, iodoacetate, ethacrynate, 3-chloroethacrynate and dihydroethacrynate (not thiol reagent).

Figure 3 shows that NEM, ethacrynate and 3-chloroethacrynate strinkingly decrease the content of free reduced glutathione while DTNB, CPDS, PCMB, mersalyl give only slight effect. Iodoacetate decreases about half the content of GSH, dihydroethacrynate (not thiol reagent) has no effect. In all experiments the amount of oxidized glutathione is not significantly modified. There is no significant variation in our

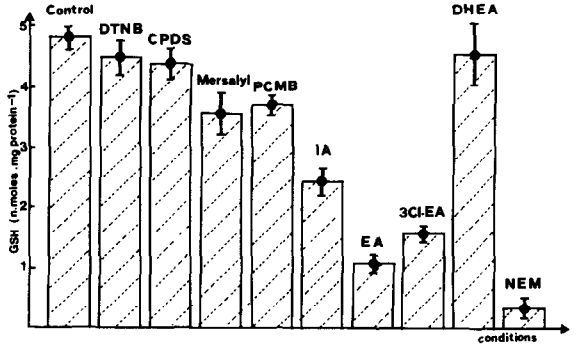

Fig.3. Free reduced glutathione content in rat liver mitochondria incubated with different thiol reagents. Experimental conditions see fig.1. Incubation time 10 min; thiol reagents, $2 \cdot 10^{-5}$ M.

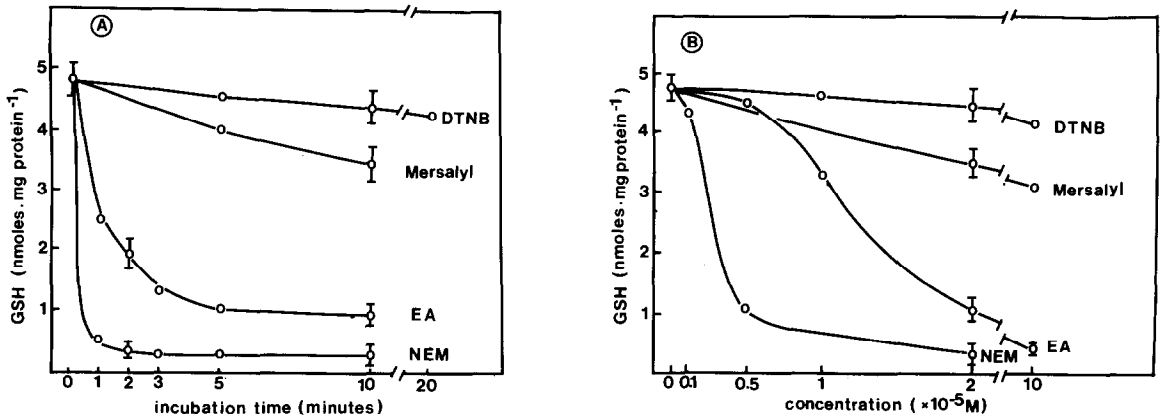

Fig.4. Influence of incubation time (A) and thiol reagent concentration (B) on the reaction between mitochondrial GSH and different thiol reagents. Experimental conditions see fig.1. A: Thiol reagents concentration $2 \cdot 10^{-5}$ M B: Incubation time: DTNB and mersalyl 10 min; NEM and ethacrynate 2 min.

results whatever the respiratory state is.

Figure 4A shows that NEM and ethacrynate penetrate readily into the matrix (NEM being much faster than ethacrynate) while mersalyl and DTNB are very poor penetrants. Figure 4B shows the influence of reagents concentration on the penetration and confirms the preceding results.

It has been checked by optical density measurement and by electron microscopy (not shown) that, in our conditions, both NEM and ethacrynate do not induce any mitochondrial swelling. This point is important since the swelling could have permitted the reaction between these reagents and intramitochondrial GSH.

These results show clearly that there are two types of thiol reagents, those which cannot react with the rat liver intramitochondrial GSH; DTNB, CPDS, mersalyl, PCMB because non-penetrant, and those which react more or less quickly with GSH: NEM, ethacrynate, 3-chloro-ethacrynate and iodoacetate, these are penetrant thiol reagents. This work confrims previous results obtained with CPDS [12] and ethacrynate [13–15] on different mitochondrial functions. Vignais et al. [7,8] have shown that fuscin and avenaciolide are penetrant thiol reagents whereas ASPM (*N*-(*N*-acetyl-4-sulfamoylphenyl)-maleimide) is a non-penetrant thiol reagent.

Table 1
Inhibition of D(-)-β-hydroxybutyrate in rat liver mitochondria by thiol reagents

Conditions	Intact mitochondria		Sonicated mitochondria		
	Oxidation rate	Inhibition %	Oxidation rate	Inhibition %	Δ % inh.
Control	10.75		43		
DTNB $8 \cdot 10^{-5}$ M	8.9	18	5.4	87	+ 69
DTNB $1.6 \cdot 10^{-4}$ M	8.4	22	7.15	84	+ 62
CPDS $4 \cdot 10^{-5}$ M	9.8	10	7.2	83	+ 73
CPDS $2 \cdot 10^{-4}$ M	9.6	12	4.3	90	+ 78
Mersalyl $2 \cdot 10^{-5}$ M	8.85	18	12.5	71	+ 53
NEM $2.4 \cdot 10^{-5}$ M	7.35	32	26	40	+ 8
EA $8 \cdot 10^{-4}$ M	5.2	52	3.6	82	+ 30

Mitochondria (2.8 mg protein) are incubated at 30°C in the respiratory medium pH 7.4 described by Ernster [6]. BOH, 4 mM. Intact mitochondria: ADP, 0.16 mM; Pi, 4 mM. Sonicated mitochondria: NAD, 0.4 mM. Oxidation rate is expressed in n·atomes·g $O_2 \cdot min^{-1} \cdot mg\ protein^{-1}$.

The existence of two types of thiol reagents, penetrant and non-penetrant, allowed us to localize the rat liver mitochondrial D(-)-β-hydroxybutyrate dehydrogenase (EC 1.1.1.30), this enzyme is tightly bound to the inner membrane [16] and very reactive SH-groups are present in the active center [17].

Table 1 shows the inhibition of D(-)-β-hydroxybutyrate oxidation by thiol reagents in intact and sonicated mitochondria. DTNB, CPDS, and mersalyl poorly inhibit the oxidation in intact mitochondria whereas in sonicated mitochondria the inhibition is very strong. NEM and ethacrynate inhibit the oxidation in both types of mitochondria. These results show that the D(-)-β-hydroxybutyrate dehydrogenase is located on the matrix face of the inner mitochondrial membrane. This conclusion is confirmed with the experiment (fig.5) where NADH oxidation is induced by acetoacetate, it is known that acetoacetate penetrates easily in mitochondria [18] whereas NADH cannot penetrate [19].

As can be seen, acetoacetate can oxidize NADH only in sonicated mitochondria, if the enzyme was located outside the inner membrane NADH would be oxidized by acetoacetate in intact mitochondria. The addition of cholate to intact mitochondria induces NADH oxidation, in these conditions, the inner membrane being more or less destroyed, the enzyme is accessible.

All these results suit with the localization of D(-)-β-hydroxybutyrate dehydrogenase on the matrix face of the inner membrane.

The existence of penetrant and non-penetrant SH-reagents appears of great interest, because, as far as an enzyme exhibits essential SH-groups, it should be possible, using the same approach, to localize this enzyme in different cytoplasmic organelles containing glutathione.

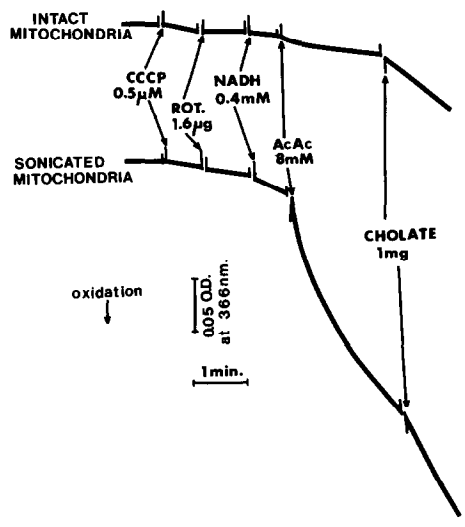

Fig.5. NADH oxidation by acetoacetate in intact and sonicated mitochondria. Experimental conditions: intact and sonicated mitochondria (2.8 mg proteins) are incubated at 30°C in the medium (2.5 ml) pH 7.4 described by Ernster [6]. Intramitochondrial NADH is oxidized by addition of CCCP, the respiratory chain is blocked with rotenone.

Acknowledgement

This work was supported by grants from the D.G.R.S.T. (contrat n° 73.7.1518) and the C.N.R.S. (E.R.A. No. 266).

References

[1] Gautheron, D. C. (1973) Biochimie 55, 727–745.
[2] Weinbach, E. C. (1961) Anal. Biochem. 2, 335–343.
[3] Jacobs, E. E., Jacob, M., Sanadi, D. R. and Bradley, L. B. (1956) J. Biol. Chem. 223, 147–156.
[4] Tietze, F. (1969) Anal. Biochem. 27, 502–522.
[5] Ellman, G. (1959) Arch. Biochem. Biophys. 82, 70–77.
[6] Ernster, L. (1960) 1st IUB/IUBS Intern. Symp. Stockholm., Biological Structure and Function 2, pp. 139–168 (Goodwin, T. W. and Lindberg, O., eds.) Acad. Press., London–New York.
[7] Vignais, P. M. and Vignais, P. V. (1973) Biochim. Biophys. Acta 325, 357–374.
[8] Vignais, P. M., Chabert, J. and Vignais, P. V. (1974) 9th FEBS Meeting, Budapest, 25–30 August, abstract No. $S_6 S_7$, p. 206.
[9] Jocelyn, P. C. (1972) in: Biochemistry of the SH-groups, pp. 223–239, Acad. Press, New York.
[10] Riley, M. V. and Lehninger, A. L. (1964) J. Biol. Chem. 239, 2083–2089.
[11] Jocelyn, P. C. and Kamminga, A. (1974) Biochim. Biophys. Acta 343, 356–362.
[12] Sabadie-Pialoux, N., Abou-Khalil, S. and Gautheron, D. C. (1972) C.R. Acad. Sci., 275, 637–640.
[13] Gaudemer, Y. and Foucher, B. (1967) Biochim. Biophys. Acta 131, 255–264.
[14] Foucher, B., Geyssant, A., Goldschmidt, D. and Gaudemer, Y. (1969) Eur. J. Biochem. 9, 63–69.

[15] Foucher, B. and Gaudemer, Y. (1971) FEBS Lett. 13, 95–97.
[16] Lehninger, A. L., Sudduth, M. C. and Wise, J. B. (1960) J. Biol. Chem. 235, 2540–2455.
[17] Latruffe, N. and Gaudemer, Y. (1974) Biochimie 56, 435–444.
[18] Chappell, J. B. (1968) Brit. Med. Bull. 24, 150–157.
[19] Klingenberg, M. and Von Jagow, G. (1970) in: Electron transport and energy conservation (J. M. Jager et al., eds.) pp. 281–290, Adriatica editrice Bari.